Compound screening in cell-based models of tau inclusion formation: Comparison of primary neuron and HEK293 cell assays

Received for publication, August 7, 2019, and in revised form, February 4, 2020. Published, Papers in Press, February 7, 2020, DOI 10.1074/jbc.RA119.010532


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The hallmark pathological features of Alzheimer’s disease (AD) brains are senile plaques, comprising β-amyloid (Aβ) peptides, and neuronal inclusions formed from tau protein. These plaques form 10–20 years before AD symptom onset, whereas robust tau pathology is more closely associated with symptoms and correlates with cognitive status. This temporal sequence of AD pathology development, coupled with repeated clinical failures of Aβ-directed drugs, suggests that molecules that reduce tau inclusions have therapeutic potential. Few tau-directed drugs are presently in clinical testing, in part because of the difficulty in identifying molecules that reduce tau inclusions. We describe here two cell-based assays of tau inclusion formation that we employed to screen for compounds that inhibit tau pathology: a HEK293 cell-based tau overexpression assay, and a primary rat cortical neuron assay with physiological tau expression. Screening a collection of ~3500 pharmaceutical compounds with the HEK293 cell tau aggregation assay, we obtained only a low number of hit compounds. Moreover, these compounds generally failed to inhibit tau inclusion formation in the cortical neuron assay. We then screened the Prestwick library of mostly approved drugs in the cortical neuron assay, leading to the identification of a greater number of tau inclusion inhibitors. These included four dopamine D2 receptor antagonists, with D2 receptors having previously been suggested to regulate tau inclusions in a Caenorhabditis elegans model. These results suggest that neurons, the cells most affected by tau pathology in AD, are very suitable for screening for tau inclusion inhibitors.

Several neurodegenerative diseases, collectively referred to as tauopathies, are characterized by the presence of intracellular inclusions composed of polymeric fibrils of hyperphosphorylated tau protein within the brain (1, 2). These tau aggregates are largely found in neurons, where they are referred to as neurofibrillary tangles (NFTs) within the soma and as neuritic threads (NTs) when in dendritic processes. AD is by far the most prevalent tauopathy, afflicting >5 million individuals in the United States (3). Whereas tau inclusions are the primary neuropathological feature in frontotemporal lobar degenerative (FTLD) disorders such as progressive supranuclear palsy, corticobasal syndrome, and Pick’s disease (2), individuals with AD also have senile plaque pathology. These extracellular plaques are composed of Aβ peptides, and a prevailing hypothesis in AD research is that Aβ plaques trigger events that ultimately lead to the development of tau pathology and consequent neurodegeneration (i.e. the amyloid cascade hypothesis) (4, 5). The belief that tau inclusions cause neurodegeneration is supported by genetic data showing inherited forms of FTLD result from tau mutations (6, 7) and the strong correlation between the extent of tau pathology and cognitive status in AD (8, 9). The linkage of tau pathology to AD symptomatology is further supported by recent studies showing that AD cognitive status (10) and brain atrophy (11) are correlated with tau PET signal. In fact, a prospective study in which Aβ plaque and tau pathology were both assessed with PET ligands confirmed that cognitive decline was closely associated with tau inclusions, and not plaque changes (12).

Tau is normally a microtubule (MT)-associated protein that appears to affect MT dynamics in axons (13, 14) and may also modulate MT interactions with molecular motors such as kinesin and dynein (15, 16). In humans, tau exists as six alternative-spliced isoforms, with either 3 or 4 MT-binding repeats and 0, 1, or 2 N-terminal alternatively-spliced exon sequences (2). Tau becomes hyperphosphorylated in all tauopathies, with...
increased phosphorylation promoting tau disengagement from MTs (17–19) with subsequent misfolding into fibrillar structures that deposit as inclusions. The tau fibrils are hypothesized to mediate a gain–of–function toxicity, and a reduction of tau binding to MTs likely also leads to increased MT dynamicity and altered axonal transport (20, 21) that may contribute to neuronal dysfunction. There is increased interest in developing tau-directed drugs for the treatment of AD and related tauopathies (22, 23), spurred in part by multiple Phase 3 clinical failures of therapeutic candidates designed to lower Aβ levels and/or plaque burden in AD brain. Moreover, there is growing recognition that abundant Aβ pathology forms a decade or more before cognitive symptoms in AD, whereas the development of robust cortical tau pathology is more proximal to symptom onset (24, 25). To date, only a small number of tau-directed drugs have progressed to clinical testing, with the majority being immunotherapeutics (26). Accordingly, there is continued need to identify new small-molecule drug candidates directed toward targets that lead to reduced tau pathology.

A limitation in identifying candidate molecules to reduce tau inclusion formation has been a paucity of robust cell-based assays that model the events leading to tau inclusion formation and clearance. Although we (27, 28) and others (29–31) have previously conducted screens to identify inhibitors of recombinant tau fibril formation, such cell-free assays do not replicate the processes involved in tau fibril formation and degradation within a cellular milieu. There would thus be considerable value in identifying cell-based models of tau inclusion formation that are suitable for compound screening. Although cellular assays of tau inclusion formation have been described and in some cases selectively queried with test compounds (32–36), they have not generally been used for extensive small-molecule screening. An exception is a recent screening of 1649 compounds conducted with the N2A cell line that overexpressed a pro-aggregant repeat domain of tau, leading to the identification of several inhibitors of tau aggregation (37). As described here, we have optimized a unique HEK293 cell assay of tau inclusion formation, as well as a primary rat cortical neuronal assay with AD-like tau pathology, and we show here that both are suitable for compound screening. Notably, the neuronal model shows a time-dependent formation of inclusions composed of endogenously expressed rat tau proteins that elongate from internalized tau “seeds” composed of enriched pathological tau derived from human AD brains (38). This neuronal assay does not rely on overexpression of mutant tau, as has been typically required to induce inclusions in cell lines or iPSC neurons (32, 33, 37, 39), and thus it provides a pathophysiologically-relevant model system in which to survey for inhibitors of tau inclusions. As described below, the National Center for Advancing Translational Sciences (NCATS) Pharmaceutical Collection compound library was screened in the HEK293 cell tau aggregation assay. Confirmed hits from this screen underwent testing in the neuronal assay of tau inclusion formation, yielding a low confirmation rate. This led to a subsequent screening of the Prestwick library of mostly approved drugs in the neuronal tau inclusion assay, which provided a higher number of validated hits with greater potential biological relevance. These findings suggest the importance of assessing compounds in neuronal models that express physiological levels of tau.

**Results**

**Compound screening in a HEK293 cell assay of tau inclusion formation**

Our laboratories previously demonstrated (32) that intracellular tau inclusions can be promoted by the cytosolic delivery of pathological seeds composed of pre-formed synthetic fibrils assembled from recombinant tau (tau pre-formed fibrils or PFFs) into HEK293 cells that express the longest form of human recombinant tau containing the P301L mutation found in inherited FTLD (referred to as T40PL) (40). Based on this cell-based system of tau inclusion formation, an alternative model was developed in which a HEK293 cell line was created that expresses T40PL with a C-terminal GFP tag (T40PL–GFP) to allow visualization of tau inclusions. T40PL–GFP expression in this clonal line is driven by a tetracycline-regulated promoter such that expression can be induced by addition of doxycycline (Dox) to the culture medium. Aggregates of T40PL–GFP were generated in these HEK293 cells through recombinant tau PFF transduction, and it was found that these intracellular aggregates could be propagated with high efficiency to daughter cells as long as cultures were maintained in the presence of Dox (41), resulting in a clonal line referred to here as T40PL–GFPagg cells. A prior study (41) showed that the tau aggregate burden within T40PL–GFPagg cells could be reduced by Dox withdrawal, with the amount of insoluble tau diminishing over 7 days after cessation of T40PL–GFP expression, such that only small insoluble tau aggregates remain. However, initiation of T40PL–GFP expression through re-introduction of Dox after the 7-day withdrawal period resulted in the rapid reformation of T40PL–GFP inclusions within 2 days, indicating that the remaining small tau aggregates were sufficient to “reseed” new tau aggregate growth upon re-expression of soluble tau (41).

The ability to manipulate the tau aggregate burden within T40PL–GFPagg cells led to the creation of a unique screening assay, as depicted in Fig. 1A, where compounds can be added to Dox-deprived T40PL–GFPagg cells with sparse aggregates prior to re-initiation of T40PL–GFP expression. This allows for an assessment of compound activity on tau inclusion formation or clearance. Preliminary studies revealed that soluble T40PL–GFP could be effectively cleared from tau inclusion-bearing cells with 1% hexadecyltrimethylammonium bromide (HDTA) extraction, followed by 4% paraformaldehyde (PFA) fixation, allowing for quantitative imaging of remaining detergent-insoluble T40PL–GFP (Fig. 1B). This tau aggregation model was optimized for a 1536-well plate format, and the efficiency of the HDTA/PFA extraction and fixation method in removing soluble T40PL–GFP prior to imaging were compared with an alternative method in which soluble T40PL–GFP was released from aggregate-bearing cells with the commercial CellTiter-Glo™ (CTG) reagent, with the T40PL–GFP being diluted into the surrounding medium such that it did not interfere with imaging of T40PL–GFP inclusions (Fig. 1B). The addition of the CTG reagent allows for the concurrent assessment of cell toxicity through a determination of a luminescent ATP signal (42),
although cell toxicity was ultimately determined by quantifying DAPI-stained cell nuclei that remained after cell extraction. The 1536-well T40PL–GFP cell assay was utilized to screen the NCATS Pharmaceutical Collection of ~3500 approved drugs and investigational compounds, utilizing the protocol summarized in Fig. 1A and described under “Experimental procedures.” All compounds were screened at five concentrations to generate concentration–response data for each compound, and the insoluble T40PL–GFP-integrated fluorescent signal was normalized to DAPI counts. All compounds were tested in two screening protocols, using the HDTA or CTG extraction methods, with tau aggregate inhibitory activity well-separated by at least 1 log unit from cytotoxicity. Generally, compounds that were hits when using the HDTA extraction procedure were also hits when analyzed after CTG extraction, although there were examples where differences in the inhibition curves were observed between the two extraction methods. A total of eight hits were identified from the screening of the ~3500 compound library that showed concentration-dependent inhibition of tau inclusion formation with minimal toxicity with both extraction procedures (Fig. 2), for a hit rate of ~0.2%.

**Optimization of a primary rat cortical neuron assay of tau inclusion formation**

The results from the screening of the NCATS Pharmaceutical Collection demonstrated that the HEK293 T40PL–GFP cell assay is robust and suitable for assessment of compound libraries. However, a relatively low number of hits were identified. Moreover, whereas transformed cell lines such as HEK293 are convenient to use for screening, they differ considerably from neurons, which are the cell type most affected with tau pathology in disease. In particular, neurons are post-mitotic, with a complex cell physiology that supports the development and maintenance of extended axonal and dendritic processes. Thus, there are likely to be significant differences in the regulation of various cellular pathways, including those involved in proteostasis, in neurons relative to rapidly dividing cells. With the objective of developing a neuronal tau inclusion assay that could be utilized to verify the activity of hits from HEK293 T40PL–GFP cell screens, efforts were made to further optimize a primary neuronal assay of tau inclusion formation that was previously described (38).

Prior studies from our laboratories revealed that robust tau inclusions form in primary mouse neuron cultures after incubation with enriched preparations of insoluble pathological tau derived from AD brain tissue (AD-tau) (38). Once internalized by neurons, this AD-tau acted to seed the formation of abundant insoluble tau inclusions, largely within neuritic processes, that were composed of endogenous mouse tau. These insoluble tau aggregates could be readily visualized 14 days after AD-tau addition after extraction of soluble tau and staining with an antibody specific to rodent tau (T49) or by fractionation of neuronal homogenates and measurement of Triton X-100–insoluble tau (38). We adapted this neuron assay to a 384-well plate format, with culturing of mouse or rat neurons for 7 days
prior to AD-tau addition, with an additional 14–15 days in culture before assessment of tau inclusions through immunostaining and imaging. Multiple conditions were investigated during optimization of the assay, including the following: 1) determination of the extraction/fixation method that best removes residual soluble tau to provide the greatest differential of insoluble tau staining between AD-tau–treated and -nontreated neurons; 2) comparison of the amount of insoluble tau pathology formed in rat versus mouse primary neurons; 3) assessment of the AD-tau dose required to provide suitable Z’
values for compound screening without reaching saturation of pathology and loss of assay sensitivity; and 4) batch-to-batch consistency of AD-tau in inducing neuronal tau inclusions. In summary, the extraction/fixation method that provided the best insoluble tau signal-to-background readings was 1% HDTA extraction with 4% PFA fixation. Interestingly, rat cortical neurons were found to develop a greater tau inclusion burden than mouse cortical neurons (Fig. 3), and cortical neurons had greater aggregate amounts than hippocampal neurons for each species (data not shown). Based on dose-response comparisons of different AD-tau preparations, little variability was observed among AD-tau batches (Fig. 3). The results of these AD-tau dose-response analyses revealed that addition of 62.5 ng/well of AD-tau to rat cortical neurons in the 384-well format consistently yielded sufficient tau pathology to provide Z’-values of >0.5 when comparing AD-tau-treated versus nontreated neurons, while remaining in the linear range of sensitivity (Fig. 3).

Utilizing the optimized primary rat neuron assay (schematic in Fig. 4A), the available hits from the NCATS Pharmaceutical Collection screen in the HEK293 T40PL–GFPagg tau aggregate assay were evaluated at four concentrations (0.3–10 μM) in triplicate. Because the HDTA extraction and PFA fixation protocol used in the assay negatively affects neuronal morphology, a parallel set of rat cortical neuron cultures were treated with AD-tau and compound but were permeabilized with 0.1% Triton X-100 and fixed with PFA, followed by staining of neuronal nuclei (NeuN antibody) and dendritic processes (MAP2 antibody) to allow assessment of compound neurotoxicity. Somewhat surprisingly, only one of the seven tested hits from the NCATS screen (NCG0024777-04; SNC-80) showed some evidence of a concentration-dependent inhibition of tau inclusions in the neuronal assay that was not also paralleled by a corresponding compound-induced toxicity, as seen by a reduction of DAPI and/or NeuN counts, and/or a reduced MAP2 area (SNC-80 result in Fig. 4B; all other tested compounds summarized in Fig. S2). In contrast, a GSK-3β inhibitor (CHIR-99021) that was tested in the rat cortical assay based on the proposed role of GSK-3β in promoting tau hyperphosphorylation and aggregation (44, 45) routinely caused a concentration-dependent decrease in neuronal tau inclusions that was somewhat separated from toxicity measures (Fig. 4C). In addition, the BMS-265246 compound used as a control in the HEK293 T40PL–GFPagg cell assay also showed evidence of inhibition of tau inclusions in the neuronal assay at nontoxic concentrations (data not shown). Thus, these data reveal that the NCATS compounds that reduced tau aggregates in the HEK293 T40PL–GFPagg cell assay were generally not active in the neuronal assay. This may relate to the considerable differences between post-mitotic primary cortical rat neurons that express endoge-
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Compound screening in the primary rat cortical neuron assay of tau inclusion formation

Given the relatively low hit rate from the screening of the NCATS Pharmaceutical Collection in the HEK293 T40PL–GFPagg cell assay, and the poor confirmation of identified hits in the neuronal assay of tau inclusion formation, a compound screen was conducted in the neuronal assay to determine whether a higher hit rate might be observed. Compounds from the Prestwick library of 1280 mostly approved drugs were tested at 10 μM as singletons in the rat cortical neuron assay as depicted in Fig. 4A. The assay performance was good, with \( Z' \)-values \( \geq 0.5 \) typically observed across the plates. Given the low confirmation rate of the hits from the HEK293 T40PL–GFPagg cell assay screen, one potential concern was that compound degradation or metabolism might occur during the 14-day incubation period, perhaps explaining the low confirmation rate of the NCATS hits, although CHIR-99021 and BMS-265246 showed reproducible activity in the assay. Gratifyingly, compound degradation did not appear to be a significant problem, as 117 compounds were identified that caused \( >3 \) S.D. reduction of tau aggregates and \( <2 \) S.D. decrease in DAPI counts.

The confirmed hits from the cortical neuron tau inclusion screen were also assessed for untoward effects on neuronal viability and/or morphology. Neuron cultures were treated with AD-tau and 10 μM compound in triplicate as above, but underwent the milder Triton X-100 and PFA permeabilization/fixation protocol to allow visualization of NeuN-positive nuclei and MAP2-positive dendritic processes. This additional analysis revealed that a number of the hits affected dendritic outgrowth and/or reduced neuron counts by \( >2 \) S.D., thereby resulting in likely false-positive reductions in tau inclusions. After removal of these compounds, a total of 32 confirmed active and non-toxic compounds were identified from the Prestwick library (2.5% confirmed hit rate; Table S1). An examination of the reported targets of these compounds based on literature review revealed several neurotransmitter receptors and neuronal enzymes, as well as likely nonspecific hits such as topoisomerase inhibitors and antibiotics. A total of 23 of these neuronal screening hits were also within the NCATS Pharmaceutical Collection, and none showed evidence of a concentration-dependent inhibition of tau inclusions in the HEK293 T40PL–GFPagg cell assay screen (Fig. S3). Moreover, two (gefitinib and naftopidil) of the eight hits identified from the NCATS Pharmaceutical Collection screen were within the Prestwick library, and neither of these compounds were found to be active at the 10 μM concentration used in the neuronal tau inclusion screen. Thus, these results further confirm the differences in compound activity observed between the neuronal and HEK293 tau inclusion assays. After prioritizing confirmed hits by removing compounds directed to undesirable targets or those with lower overall tau aggregate inhibition on triplicate testing, 18 compounds that caused \( >3 \) S.D. inhibition of tau inclusions upon triplicate testing (highlighted in Table S1) underwent concentration–response analyses, with 13 of these showing well-defined inhibition curves with calculable IC_{50} values (Table 1).

Each of these 13 compounds was subsequently evaluated in an orthogonal assay to confirm that they reduced insoluble neuronal tau. This consisted of testing cellular lysates from AD-tau- and compound-treated rat cortical neurons, as in Fig. 4A except in 96-well plates, in a tau multimer ELISA. This ELISA utilizes a rodent tau-specific mAb as both a capture and detection antibody, such that monomeric tau is not detected due to epitope shielding after monomer binding to the capture antibody. Conversely, rodent tau multimers, including fibrils, are detected in the ELISA. Importantly, the specificity for rodent tau precludes the detection of residual AD-tau that might remain in the culture lysates. Each of the 13 tested compounds showed appreciable inhibition of multimeric tau when tested in triplicate at the \( \sim \) EC_{90} concentration determined from the concentration–response curves for the compounds (Table 1; alfuzosin tested at EC_{50}). In general, there appeared to be a greater compound-mediated inhibition of multimeric tau as measured with the ELISA than had been observed when quantifying tau aggregates via immunostaining and imaging. This could be the result of compound-mediated reduction of diffusible tau multimers that do not persist after extraction prior to immunostaining. Nonetheless, the biochemical assay confirms that the compounds listed in Table 1 indeed reduce multimeric and insoluble tau in the rat cortical neuron assay.
Among the 13 compounds with defined IC₅₀ values were a number of reported dopamine D2 receptor antagonists, all of which had well-defined concentration–response curves (Fig. 6). The inhibitory activity observed in the neuronal tau inclusion assay appears to be generally consistent with the reported binding affinities of these compounds. For example, both alizapride and metoclopramide have reported D2 receptor affinities of ~300 nM (46), which is roughly the IC₅₀ values obtained with these compounds in the neuronal tau assay. We assessed whether these D2 receptor antagonists act to reduce overall tau protein expression by measuring the amount of soluble tau by ELISA in compound-treated rat cortical neuron cultures, and these compounds did not generally affect soluble tau, although bromopride and azaperone caused modest decreases with the latter reaching statistical significance (Fig. S4). To further investigate possible mechanisms by which the D2 receptor antagonists prevent neuronal tau inclusions, we evaluated their ability to directly inhibit tau fibrillation. None of the identified D2 receptor antagonists reduced the in vitro fibrillation of recombinant tau, whereas the positive control compounds oleocanthal (47) and CNDR-51348 (48) both caused concentration-dependent inhibition of tau fibril formation (Fig. S5).

We also examined whether the identified D2 receptor antagonists might inhibit neuronal tau inclusion formation through a reduction of tau phosphorylation. It is known that tau becomes hyperphosphorylated in AD and related tauopathies, and increased phosphorylation can elevate cytosolic tau concentrations through promotion of tau disengagement from microtubules (18, 19, 49). Moreover, phosphorylation may also increase the propensity of tau to fibrillize (50–52). Tau phosphorylation was evaluated in the standard tau neuronal assay (Fig. 4A) after treatment with vehicle or two of the active D2 receptor antagonists (metoclopramide and azaperone) that have differing core chemical scaffolds. At the end of the culture incubation period, the neurons were homogenized in RIPA buffer, and RIPA-soluble and -insoluble fractions were obtained. Tau phosphorylation in the RIPA-soluble tau was examined because it is increased phosphorylation of soluble tau that would likely enhance tau inclusion formation. Moreover, analysis of phosphorylation changes in insoluble tau is confounded by contaminating background tau phosphorylation from the added AD-tau and the fact that neuronal tau inclusions are greatly reduced by treatment with the D2 receptor antagonists (Fig. 6). Although there are >40 described tau phosphorylation sites (53, 54), we chose to specifically assess phosphorylation at Ser-199/Ser-202/Thr-205 (AT8 antibody) and Ser-396/Ser-404 (PHF1 antibody), as these sites are phosphorylated in AD brain

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**Table 1**

Summary of confirmed hits with calculable IC₅₀ values from the Prestwick library screen in the rat cortical tau inclusion assay

Data are provided showing the inhibitory activity of each compound on tau inclusions and DAPI nuclei from the original screen (10 μM compound) and tau inclusion, DAPI, MAP2 and NeuN inhibitory activity in the triplicate confirmation analyses (10 μM compound). In addition, the calculated tau aggregate IC₅₀ values and maximal inhibition from concentration–response testing are listed for each compound. Finally, the inhibitory activities of the compounds as measured in the orthogonal tau multimer (mTau8) ELISA are shown, with each compound tested at the approximate IC₅₀ concentration (except Alfuzosin, tested at IC₅₀).
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tau inclusions, with the extent of phosphorylation at these sites correlating with disease stage (55). Little to no A/β immunoreactivity was detected in the RIPA-soluble fraction of AD-tau treated neurons, suggesting that phosphorylation at this site is unlikely to contribute to the observed tau aggregation in the neuron cultures. However, PHF1-positive tau was observed in the RIPA-soluble fraction of neurons treated with or without AD-tau (Fig. S6A). Neurons that also received metoclopramide or azaperone showed no change in PHF1-positive tau relative to those treated with vehicle only (Fig. S6, A and B). Thus, it does not appear that the D2 receptor antagonists decrease tau inclusion formation in the neuronal assay through a reduction of phosphorylation at the Ser-396/Ser-404 or Ser-199/Ser-202/ Thr-205 sites. We cannot, however, exclude the possibility that the active D2 receptor antagonists alter phosphorylation at one or more of the other tau phosphorylation sites.

Interestingly, there is prior evidence of D2 receptor antagonists reducing the formation of tau inclusions in a Caenorhabditis elegans model with tau pathology, where azaperone reduced levels of insoluble tau and inhibited neurodegeneration (56). Moreover, genetic knockout of the C. elegans D2-like receptors had a similar tau aggregate-reducing effect (56). Although the detailed mechanism linking D2 receptor function and tau pathology in C. elegans is still being investigated and may be indirect (57), the ability of the neuronal tau inclusion screen to identify these compounds suggests the following: 1) this mechanism may exist in mammalian as well as C. elegans neurons, and 2) the neuronal assay of tau inclusion formation appears to be capable of identifying biologically relevant hits. In this regard, the screening of larger compound libraries in this assay may lead to the identification of additional small molecules and associated molecular targets that regulate the formation or clearance of tau aggregates.

Discussion

The amyloid cascade hypothesis of AD (4, 5) was formulated over 20 years ago based on genetic evidence pointing to a key role of A/β peptide-containing senile plaques in AD onset. As a result, the vast majority of AD drug candidates that have progressed to Phase 3 testing have been designed to reduce A/β levels and/or plaque burden, and to date all have failed to show sufficient efficacy to garner regulatory approval. Insights into the possible reasons for these clinical failures have been provided by advances in our understanding of brain changes that occur as AD progresses from prodromal to symptomatic stages. In particular, PET and magnetic resonance imaging (MRI) methodologies that allow for assessment of AD brain function and pathology, as well as cerebrospinal fluid biomarkers that provide potential information about brain changes, reveal that plaque pathology precedes symptom onset by a decade or more (24). Thus, the administration of A/β-directed drug candidates to patients with even early clinical signs of disease (i.e. mild cognitive impairment) may be ineffective because plaque pathology has largely plateaued and the cascade of events triggered by A/β accumulation have progressed to a stage where lowering of the plaque burden could be without meaningful benefit. In contrast, immunohistochemical (8, 9) and PET imaging studies (10, 12) provide strong evidence that the development of robust tau pathology in AD brain occurs well after A/β accumulation, with tau burden showing a strong correlation with patient cognitive status. These findings suggest that tau-directed therapeutics may have greater utility in early symptomatic and late prodromal AD patients than those targeting A/β, and there has been increased interest in the pharmaceutical sector in identifying mechanisms and molecules that will reduce tau pathology.

A number of strategies have been proposed to reduce the development or consequences of tau pathology (58), but to date most have not resulted in drug candidates entering clinical testing. There are presently multiple immunotherapy trials ongoing in which tau antibodies are being assessed for their ability to slow AD progression (26). In addition, there have been pharmaceutical programs directed to the inhibition of kinases thought to play a role in the hyperphosphorylation of tau (59–61), but this approach has not resulted in clinical success. Finally, the compound LMTX® that was proposed to act as a tau fibrillization inhibitor (62) failed to achieve the co-primary end points in a Phase 3 study in AD patients. There would clearly be value in taking a relatively unbiased approach to identifying new small molecules and associated targets that modulate tau inclusions. In this regard, the development of robust cellular assays that mimic the processes of tau inclusion formation and turnover would be important. Although such assays have been previously developed using cell lines in which mutated and/or truncated tau proteins were overexpressed (32–36), only very recently has such an assay been utilized for extensive compound screening (37). In addition, a model of tau aggregation has been described in human iPS cell-derived neurons that overexpress tau (39), although compound screening was not reported. We report here on two assays of fibrillar tau aggregate formation: one in HEK293 cells that overexpress GFP-tagged T40 tau harboring the P301L mutation found in familial FTLD (41), and the other in primary rat cortical neurons in which fibrillar tau inclusions develop from endogenously expressed rat tau after seeding of the cultures with AD-tau (38). The HEK293 cell assay utilizes cells that maintain intracellular tau aggregates (T40PL–GFP® cells), and these aggregates can be reduced in number and size by inhibiting tau expression from a tetracycline-inducible promoter through Dox withdrawal. The tau aggregate reformation can be triggered upon re-initiation of tau expression in the T40PL–GFP® cells, and the addition of compounds prior to tau re-expression allows for the identification of molecules that affect tau inclusion formation or clearance.

The screening of the NCATS Pharmaceutical Collection in the T40PL–GFP® cell assay resulted in a low hit rate, which could reflect a difficulty in finding molecules that inhibit tau fibrillization or tau aggregate clearance in a cellular milieu. Alternatively, it could be that the T40PL–GFP® cells, which were selected based on their ability to be propagated while harboring tau aggregates, have an altered cellular physiology that renders them relatively insensitive to compounds that might alter the generation or clearance of tau inclusions. Disappointingly, only one hit from the NCATS Pharmaceutical Collection screen (SNC-80; opioid receptor agonist) showed some evidence of activity in the primary rat cortical assay of tau aggre-
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HEK293 T40PL–GFP<sup>agg</sup> cell 1536-well compound screening

HEK293 T40PL–GFP<sup>agg</sup> cells were maintained in DMEM (Thermo Fisher Scientific, catalog no. 11995) + 10% tetracycline-screened FBS (HyClone), 100 units/ml penicillin, 100 μg/ml streptomycin, 1% Glutamax, 5 μg/ml blasticidin, and 200 μg/ml Zeocin + 100 ng/ml doxycycline. For use in compound screening, HEK293 T40PL–GFP<sup>agg</sup> cells were cultured in DMEM + 10% tetracycline-screened FBS + 1% penicillin-streptomycin + 1% GlutaMAX for 6 days (Dox removal stage). Cells were plated into a 1536-well black clear-bottom cyclic olefin imaging plate (Aurora Microplates) at 400 cells per well in a 6.5-μl volume using a Multidrop Combi dispenser (Thermo Fisher Scientific). Plates were either standard tissue culture–treated (when using CellTiterGlo extraction protocol) or coated with poly-D-lysine (hexadecyltrimethylammonium (HDTA) extraction protocol). After overnight incubation, compounds were added at five concentrations (110 nM to 27.5 μM) in singlets using a pin transfer tool (Wako), and 1 μl of 750 ng/ml doxycycline, prepared in DMEM described above, was added to each well (final concentration 100 ng/ml). Control wells without Dox treatment (1 μl DMEM addition) were included on each plate. Cells were returned to a humidified incubator for 48 h. For the CellTiterGlo extraction protocol, 2 μl of CellTiterGlo luminescent cell viability reagent (Promega) containing 10 μg/ml Hoechst 33342 was added to each well, and plates were incubated at room temperature for 5 min. Data were collected using an IN Cell2200 automated wide-field imager at ×10 magnification to acquire the entire well from a single field of view image. The system utilized laser-based auto-focus and standard filters to capture tau-GFP (excitation 475/28 and emission 512/23) and Hoechst (excitation 390/18 and emission 432/48). Cell viability was assessed by reading luminescence on a ViewLux Microplate Imager (PerkinElmer Life Sciences) equipped with clear filters. For the HDTA extraction protocol, angled aspirators and dispensers were used for all medium exchanges. First, cells were washed five times by sequentially aspirating medium (leaving 4 μl in well) and adding 4 μl of PBS to each well. On the final wash, 4 μl of PBS remained in the well, and an additional 4 μl of PBS + 2% HDTA was added. Cells were incubated at room temperature for 10 min, and medium was aspirated to a volume of 4 μl per well, followed by addition of 4 μl of 8% paraformaldehyde (prepared in PBS). Plates were incubated at room temperature for 20 min, and five aspiration/wash steps (4 μl) were performed using PBS + 0.05% Tween 20. Then, 4 μl of PBS + 0.05% Tween 20 + 10 μg/ml Hoechst 33342 was added to each well, and plates were...
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incubated at room temperature for 20 min and imaged on an InCell2200 as outlined above. Fluorescence intensity was quantified using InCell Developer Toolbox (GE Healthcare), with intensity and area of large aggregates (4–10 μm diameter size criterion) calculated. Nuclei were identified by using standard top hat segmentation with a size criteria of ~75 μm in diameter. For assessing compound activity, the feature “sum fluorescence intensity × area of large aggregates” was normalized to cell number (nuclei counts, Hoechst 33342 staining). The screening data were analyzed using software developed internally at NCATS. Percent activity of compounds was normalized to Dox + vehicle (0% activity) and No-Dox (~100% activity) controls on a per-plate basis. The same controls were used for the calculation of the Z’ factor index for each assay. Compound activity was fitted to the Hill equation using in-house software (NCGC CurveFit, RRID:SCR_018079), and concentration–response curves were generated. Active compounds had defined dose-dependent inhibition of tau aggregation and activity in both HDTA and CTG assays. Visual inspection of curves was performed to ensure compound activity.

Human detergent-insoluble tau (AD-tau) extracts

Detergent-insoluble tau (AD-tau) was prepared essentially according to Guo et al. (38). An intermediate fraction (termed the 1% Sarkosyl pellet in Ref. 38) was determined to be suitable for primary neuron transduction as long as the AD-tau purity was ≥10% of total protein as measured by Tau5 ELISA (below) and BCA (Pierce) protein assays. Sporadic AD cases used to prepare AD-tau were pre-screened to ensure high neuritic tau burden in the frontal cortex.

Rat cortical neuron tau inclusion assay

Primary rat cortical neurons were obtained from a core facility at the University of Pennsylvania and prepared according to previously published methods (66). The cell suspension was plated on poly-d-lysine–coated 384-well plates at 5000 viable cells per well in plating medium (complete Neurobasal medium (Gibco) with 5% FBS), which was replaced at 1 day after plating with complete Neurobasal medium supplemented with 1% penicillin–streptomycin (Gibco), 1% GlutaMAX (Gibco), 2% B-27 supplement (Gibco). The plated neurons were treated with compound and were transduced with AD-tau after 7 days in culture, replacing 50% of the medium with 1:1 preconditioned neuronal medium/fresh complete Neurobasal medium, followed by a 14–15-day incubation period in which cultures were not disturbed. Cells were then extracted and fixed for assessment of insoluble tau formation or fixed and permeabilized for morphology and toxicity assessment. Wells used for assessment of neuronal morphology by fixation–permeabilization received 25 μl of 8% PFA in PBS, followed by removal of 50% of the solution (25 μl) and addition of 25 μl of 0.25% Triton X-100. After blocking, wells were incubated overnight with primary antibody, T49 (mouse anti-rodent tau monoclonal (67)) for extracted cells, and 17028 (rabbit anti-MAP2, (68)) for dendrite outgrowth and anti-NeuN (MAB377; Millipore) for neuron counts for fixed–permeabilized cells. Fluorescently-labeled secondary antibodies (Invitrogen goat anti-mouse 488 and goat anti-rabbit 594) were used for visualization, and DAPI (0.4 μg/ml) was added with secondary antibodies to stain cell nuclei. Plates were imaged on an INCell2200 (GE Healthcare) with a ×10 objective, using nine 1024 × 1024 pixel images per well for 56% coverage of the center of the well to avoid edge fixation artifacts. Data were collected in two wavelengths from HDTA-extracted plates, with nuclear signal (INCell2200 DAPI channel) and mouse tau (INCell2200 FITC channel), or three wavelengths for fixed–permeabilized plates, with nuclear signal (INCell2200 DAPI channel), NeuN (INCell2200 FITC channel), and MAP2 (INCell2200 Texas Red channel). Images were quantified using InCell Developer Toolbox (GE Healthcare). DAPI counts were determined by object size segmentation using kernel size 15 and sensitivity of 1. Then tau staining and MAP2 staining were gated by object intensity. NeuN counts were determined using object intensity followed by clump breaking using a DAPI reference. Neuronal tau inclusion burden was expressed as T49 (area × optical density)/DAPI count. Compound activity was plotted as percent inhibition relative to vehicle-treated AD-tau–transduced control wells, with subtraction of background signal derived from nonAD-tau–treated wells. Compound-mediated toxicity was monitored by three measures: DAPI counts, MAP2 area/DAPI count, and NeuN counts, all expressed as a percent of vehicle-treated AD-tau–transduced control.

Assessment of neuronal multimeric tau

Neuronal cultures that were utilized to determine the extent of compound-mediated reduction of multimeric tau as determined by ELISA were plated on 96-well plates at 17,500 cells/well in 100 μl of plating medium (as above), with medium replacement after 1 day to complete neurobasal medium. AD-tau transduction was scaled based on the fibril concentration from the 384-well plate to 2.5 μg/well (1.25 ng/μl). Timing of AD-tau and compound addition was as described for the 384-well plate assay. Following 14–15 days of incubation after AD-tau addition, the plated cells were extracted with 50 μl of 0.1% RIPA (50 mM Tris, pH 8.0, 150 mM NaCl, 5 mM EDTA, 0.5% sodium deoxycholate, 1% Nonidet P-40, and 0.1% SDS) after washing the wells with PBS on the BioTek ELx405 plate washer, fully evacuating the wells with a hand-held aspiration wand and then addition of 50 μl/well of RIPA, pH 8.0, with extensive trituration. Maxisorp plates (Thermo Fisher Scientific) were coated with 2.5 μg/ml mTau8 IgG fraction (mouse anti-rodent tau, kindly provided by Janssen Pharmaceutica) and blocked with 1% Blockace in PBS until needed. Cell RIPA extracts were diluted 5-fold in 0.2% BSA in PBS to achieve a linear range of detection and added to the ELISA plates, with overnight incu-
bation. Plates were washed in PBS and then incubated in biotinylated mTau8 followed by incubation with streptavidin–horseradish peroxidase conjugate. Visualization was done using TMB A+B reagents and stopping with 10% phosphoric acid. Plates were read at A$_{450}$ and data were normalized to BCA protein content and plotted as percent inhibition relative to fibril-transduced vehicle controls.

**Soluble tau ELISA**

Neuronal cultures were grown on 96-well plates and treated with AD-tau and compounds as described for the tau multimer ELISA. These underwent the ELISA conditions as described above, except that plates were coated with 2.5 μg/ml Tau5 IgG fraction (mouse anti-pan tau, Thermo Fisher Scientific), and the detection antibodies were a mixture of biotinylated IgG fraction (mouse anti-pan tau, Thermo Fisher Scientific), and then transduced with control medium or 1.25 μM BT2 and HT7 (Thermo Fisher Scientific, 31.2 and 62.5 ng/ml respectively).

**Phospho-tau analysis**

Rat cortical neurons were cultured as in the 384-well plate tau inclusion assay, but scaled for 12-well plates (Costar) resulting in 100,000 cells per well (26,000 cells per cm$^2$) in 1 ml of plating medium. Cultures were grown for 7 days and then treated with metoclopramide or azaperone at concentrations selected from concentration–response data to result in maximal inhibition (1 μM final concentration for both compounds) or 50% inhibition (50 nM for metoclopramide and 300 nM for azaperone). Plates were incubated for 30 min with compound and then transduced with control medium or 1.25 μg of sonicated AD-tau fibrils. After an additional 15 days in culture, cells were washed with ice-cold PBS and then scraped in 100 μl of RIPA buffer per well and sonicated to ensure disruption of cellular contents. Sonicated samples were centrifuged at 100,000 × g for 30 min. Pellets were resuspended in 100 μl of RIPA as supernatants and sonicated with 20 pulses to fully disperse the pellet material. Compound inhibition of tau inclusion formation was confirmed for the neurons receiving 1 μM D2 receptor antagonist and AD-tau by analysis of aliquots from the pellet fraction in the mTau8 rodent tau-specific multimer ELISA.

Aliquots from the RIPA homogenate supernatant fraction were run on 10% polyacrylamide gels (Bio-Rad Protein III) and then blotted onto 0.22-μm pore size nitrocellulose (Bio-Rad). Blots were blocked using LiCOR blocking buffer for 1 h at room temperature and then incubated in primary antibody overnight. Mouse tau was identified using an in-house rabbit polyclonal anti-roden tau antibody (R2295M) that had been preadsorbed to remove antibody recognizing human tau (38). Phospho-tau was identified with AT8 (Thermo Fisher Scientific; MN1020) mAb or PHF1 mAb (prepared in-house). Phospho-tau signal was quantified on a LiCOR imager and normalized to the mouse tau signal, utilizing Image Studio software with local background signal correction. All treatments were run in triplicate wells, with each of the triplicate samples run on separate gels such that each experimental condition was represented once on each gel.

**References**


Identification of tau aggregate inhibitors


Identification of tau aggregate inhibitors


